Ecotoxicology and Environmental Safety 157 (2018) 409–416

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

# Interactive effect of climate factors, biochar and insecticide chlorpyrifos on methane consumption and microbial abundance in a tropical Vertisol

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ARTICLE INFO

*Keywords:*
Elevated $CO_2$
Temperature
Biochar
Chlorpyrifos
$CH_4$
16S rRNA

ABSTRACT

Climate change may increase the pest infestation leading to intensive use of insecticides. However, the effect of insecticide and climate factors on soil methane ($CH_4$) consumption is less understood. A laboratory experiment was carried out to evaluate the effect of temperature (15 °C, 35 °C, and 45 °C), moisture holding capacity (MHC) (60%, 100%), biochar (0%, 1%) and chlorpyrifos (0 ppm, 10 ppm) on $CH_4$ consumption and microbial abundance in a tropical Vertisol of central India. Methane consumption rate k (ng $CH_4$ consumed $g^{-1}$ soil $d^{-1}$) varied from 0.065 ± 0.005 to 0.608 ± 0.018. Lowest k was in 15 °C-60% moisture holding capacity (MHC)-no biochar and with 10 ppm chlorpyrifos. Highest k was in 35 °C-100% MHC-1% biochar and without (0 ppm) chlorpyrifos. Cumulative $CO_2$ production (ng $CO_2$ produced $g^{-1}$ soil $d^{-1}$) varied from 446 ± 15 to 1989 ± 116. Both $CH_4$ consumption and $CO_2$ production peaked in the treatment of 35 °C-100% MHC-1% biochar. Chlorpyrifos inhibited $CH_4$ consumption irrespective of treatments. Abundance of 16S rRNA of eubacteria (× $10^6$ $g^{-1}$ soil) varied from 2.33 ± 0.58 to 85.67 ± 7.00. Abundance of 16S rRNA genes representing Actinomycetes (× $10^4$ $g^{-1}$ soil) varied from 7.67 ± 1.53 and pmoA gene (Methanotrophs) (× $10^5$ $g^{-1}$ soil) varied from 1.23 ± 0.59 to 34.33 ± 6.51. Chlorpyrifos inhibited abundance of heterotrophic bacteria and methanotrophs but stimulated actinomycetes. Biochar stimulated the $CH_4$ consumption, $CO_2$ production and microbial abundance. Study highlighted that use of chlorpyrifos under climate change factors may inhibit $CH_4$ consumption but the use of biochar may alleviate the negative effect of the chlorpyrifos.

## 1. Introduction

Climate change will affect soil ecosystem by impacting the microbial biomass, diversity, and their metabolic activities (Romero-Olivares et al., 2017). Therefore, understanding the function of microbes in response to the global change is important for maintenance of ecosystem. It is also predicted that use of insecticide will increase many fold in future because the prevalence of pests will increase under elevated atmospheric $CO_2$ and temperature (Yan et al., 2017). The insecticide chlorpyrifos (0,0-diethyl-3,5,6-trichloro-2- pyridylphosphorothioate) is a widely used for treatments of most of the crops, lawns, and ornamental plants (Gomez, 2009). This broad-spectrum insecticide is effective in controlling a variety of insects including mosquitoes (larvae and adults), flies, and ecto-parasite of cattle and sheep (Kumar1 and Kumar, 2007; Liu et al., 2005). The effects of chlorpyrifos on soil microbial biomass carbon and nitrogen, microbial activities including nitrogen cycling have been studied (Riah et al., 2014). However, little information is available on the impact of chlorpyrifos on soil methane consumption. Methane consumption is an important process for climate change perspective. $CH_4$ consumption activity of soil reduces concentration of atmospheric greenhouse gas methane. Methane is an important greenhouse gas as its global warming potential is about 25 times higher than $CO_2$ (Kollah et al., 2017). $CH_4$ concentration in different soil compartments may vary from 1000 to 20,000 ppm which is emitted to atmosphere (Metz et al., 2007). Therefore, consumption of atmospheric $CH_4$ is crucial to regulate climate change. If the chlorpyrifos applied to soil inhibits $CH_4$ consumption, then its use will adversely affect the ecosystem. Therefore, strategies need to be identified to minimize the negative effect of chlorpyrifos on $CH_4$ consumption. However, there is lack of information on the impact of chlorprifos on $CH_4$ consumption. It is also not clearly known how chlorpyrifos influences $CH_4$ consumption under the influence of climate factors (temperature and moisture).

It is estimated that at the current pace of $CO_2$ increase, even with measures to minimize $CO_2$ emissions, the atmospheric concentration of $CO_2$ will reach 550–700 ppm by 2050 and 650–1200 ppm by 2100 (Higgins et al., 2015). Due to the increase of atmospheric greenhouse gases (GHGs) the mean global temperature is predicted to rise by 2.5 °C

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https://doi.org/10.1016/j.ecoenv.2018.03.092
Received 15 August 2017; Received in revised form 16 March 2018; Accepted 30 March 2018
Available online 11 April 2018
0147-6513/ 

or more by 2050 and up to 6.4 °C by the end of this century (IPCC, 2007). Elevated temperature and $CO_2$ affects $CH_4$ consumption by reducing methanotrophs population. Elevated $CO_2$ and temperature influences certain aerobic microbial population.

Recently, biochar (BC) use in agriculture has been suggested as a potential win–win strategy for climate change mitigation and food production (Kollah et al., 2015). Biochar is a pyrolysed biomass of organic feedstock, produced by heating in an oxygen-limited environment (pyrolysis) at temperature of 400–900 °C (Lehmann, 2007). High cation exchange capacity (CEC) of BC enables it to absorb $NH_4$-N and other plant nutrients (Subedi et al., 2013). Biochar may also absorb various insecticides and reduce their toxicity (Diez et al., 2013). In addition biochar may enhance microbial groups and stimulate insecticide biodegradation (Atkinson et al., 2010).

Rise in temperature is likely to increase the water retention potential of atmosphere. This may lead to intense precipitation during wet season. Therefore, up-lands of tropics is expected to remain submerged in future climate. Thus, apart from increasing atmospheric $CO_2$, climate variables like temperature and moisture will vary in future climate. Considering these facts, experiments were carried out to evaluate the influence of chlorpyrifos on soil $CH_4$ consumption and abundance of different microbial groups under the influence of temperature and moisture. Experiments were also undertaken to study the role of biochar on the effect of chlorpyrifos on $CH_4$ consumption in a Vertisol.

## 2. Materials and methods

### 2.1. Study site

Study site was located at the agricultural experimental field located at the Indian institute of soil science, Bhopal, Madhya Pradesh, India (23°18'N/77°24'E, 485 m above sea level) (Mohanty et al., 2015). The experimental field was maintained under national project on organic farming since 2004. Fields were planted with soybean (*Glycine max* L.) and wheat (*Triticum aestivum* L.) during the summer and winter seasons, respectively. Wheat variety HI 8498 and soybean variety JS 335 were grown at a spacing (cm) of 22.5 × 5 and 45 × 5, with seeding rates of 100 kg and 80 kg $ha^{-1}$ respectively. Soils were collected during 2015 from the soybean field that received no fertilizer inputs. Sampling was done at the vegetative growth phase of soybean (45 days after sowing). A composite sample was prepared for this experiment by mixing 4 samples from corners and 1 sample from centre of the field. Soil were collected from 5 to 15 cm depth profile. The location has a humid subtropical climate, with a hot summer and a humid monsoon season. It experiences southwestern monsoon rains between July and September. Mean annual temperature remains about 25 °C. Highest temperature reaches near 45 °C during the mid summer (May–June). During winter (December–January) the average temperature remains about 15 °C, the average yearly precipitation is 1200 mm and air humidity is 65%.

### 2.2. Soil physico-chemical properties

The soil is a heavy clayey Vertisol (Typic Haplustert) and the experimental site was characterized with 5.7 g organic C, 225 mg available N, 2.6 mg available P, and 230 mg available K. Organic carbon (OC) was determined by wet digestion method (Walkley and Black, 1934). Available N was determined by alkaline $KMnO_4$ method (Subbiah and Asija, 1956). Available phosphorus was extracted by 0.5 N $NaHCO_3$ solution buffer at pH 8.5 (Olsen, 1954) and phosphorus in the extract was determined by ascorbic acid method (Watanabe and Olsen, 1965). Available potassium was extracted by shaking with neutral normal ammonium acetate for 5 min (Hanway and Heidel, 1952) and then K in the extract was determined by flame photometer (Lindsay and Norvell, 1978).

The electrical conductivity (EC) was 0.43 dS $m^{-1}$ and the pH was 7.5 (1:2.5 of soil and water in w:v) (Smith and Doran, 1996). The mean weight diameter of soil aggregates was 0.53 mm, and total, micro and macro porosity values were 51.7%, 32.1% and 19.6%, respectively (Yoder, 1936). The water holding capacity, bulk density and saturated hydraulic conductivity of the soil were 62% (w/w), 1.45 mg $m^{-3}$, and $7.3 \times 10^{-6}$ m $s^{-1}$ respectively. The textural composition of soil was: sand 15.2%, silt 30.3%, clay 54.5%.

## 3. Biochar preparation

The biochar (BC) was produced by slow pyrolysis from the stalks of pigeon pea (*Cajanus cajan*) grown in the experimental farm located at Central Institute of Agricultural Engineering Institute, Bhopal, India. The sun dried of pigeon pea stalks were shredded to 5–7 cm in length. The system used for BC generation was an unconfined insulated chamber made of mild steel. The chamber had inner diameter of 360 mm, height of 500 mm and wall thickness of 2 mm. Chamber temperatures was maintained externally and the heating rate to attain the pyrolysis process temperature (450 °C) was 6 °C $min^{-1}$. Charring process took about 4 h. The details about the BC unit and thermal degradation behaviour of char are given elsewhere (Gangil, 2014). Characterization of biochar was estimated by standard protocols (Nelson and Sommers, 1982). The pH (1:1.25, $H_2O$), electrical conductivity, ash content and bulk density of the BC were 9.57, 1.95 (dS $m^{-1}$), 15.5% and 239 kg $m^{-3}$ respectively. The total C, N, P and K concentrations (%) of BC were 86.4, 0.40, 0.09, and 0.74 respectively. Total Ca, Mg, and Na concentration (mg $kg^{-1}$) was 92.2, 19.5, and 395 respectively. The BC was grounded manually and passed through 2 mm sieves to achieve particle size < 2.00 mm.

## 4. Experimental setup and soil incubation studies

Temperature of the location goes down to an average of 15 °C during winter and peaks to 45 °C in summer. The location receives heavy rainfall during monsoon and soil remains submerged. To mimic the condition of dry and wet season, the experiment was carried out by incubating soil at 60% moisture holding capacity (MHC) and 100% MHC. Usually the 60% MHC represent the general field moisture condition and 100% MHC represents flooded condition of wet season. Chlorpyrifos is applied at different concentration depending on the crop and intensity of infestation. Biochar is applied at 1–2% as soil amendment (Asai et al., 2009). Considering these agricultural field scenarios experiment was undertaken. The experiment used a factorial design to determine the impact of different climate factors, biochar and insecticide on $CH_4$ consumption. The factors were temperature (15 °C, 35 °C, 45 °C), moisture (60%, 100%), biochar (0%, 1%) and chlorpyrifos (0 ppm, 10 ppm). Each factorial combination (3 temperature × 2 soil moisture × 2 biochar × 2 choropyrifos) was replicated 3 times, for a total of 72 experimental units. A 1000 ppm stock solution of chlorpyrifos (Sigma Aldrich, USA) was prepared using HPLC grade acetonitrile (Sigma Aldrich, USA). The chlorpyrifos stock of 0.l ml was added to 130 ml pre-sterilized serum vials. These vials represented the treatments of 10 ppm (w/w) chlorpyrifos. Similarly, the vials added with 0.1 ml pure acetonitrile served as treatment of 0 ppm chlorpyrifos. To nullify the effect of solvent on microbial activity vials were kept open for overnight to evaporate acetonitrile completely. To each vial 10 g portion of air dried soil was placed. Biochar was added to vials at the level of 0% or 1% (w/w). Sterile distilled water was added to maintain 60% or 100% moisture holding capacity (MHC). The contents of the vials were mixed thoroughly, capped with rubber septa and sealed using aluminium crimp seal. One ml of pure $CH_4$ was injected into the headspace of the vials for a final concentration of 1000 ppm. Vials were incubated at 15 °C, 35 °C or 45 °C in separate biological oxygen demand (BOD) incubators (Metrex scientific instruments Pvt. ltd., N Delhi, India). Vials were shaken at 100 rpm (rotation per minute) for 8 h per day. At regular intervals (~ 1 day), 0.1 ml of headspace gas was analyzed for $CH_4$. After each sampling, the headspace was replaced with an

equivalent amount of high purity helium (He) to maintain atmospheric pressure. The gas He was used because of its inert chemical nature. Vials were incubated for 15 days. The rate constant of $CH_4$ consumption (k) was determined from the slope of log-transformed values of $CH_4$ versus time during the rapid decline phase. $CO_2$ production was estimated by quantifying headspace $CO_2$ concentration after the end of incubation period.

### 4.1. Gas chromatography analysis

The $CH_4$ or $CO_2$ mixing ratios in the headspaces of serum bottles were analyzed using a gas chromatograph (CIC, India) equipped with an FID and a Porapak Q column (2-m length, diameter 2/8", 80/100 mesh, stainless steel column) as described elsewhere (Mohanty et al., 2017). The injector, column and detector were maintained at 120 °C, 60 °C and 300 °C, respectively. $CO_2$ was measured after conversion to methane via a methanizer at 330 °C. Under these conditions, the retention time of $CH_4$ was 1.3 min and $CO_2$ was 2.5 min. The GC was calibrated before and after each set of measurements using different mixtures of $CH_4$ in $N_2$ (Sigma Gases, New Delhi, India) and/or $CO_2$ in $N_2$ (Inox Air, Bhopal, India) as primary standards ($CH_4$/$CO_2$ 100 ppm).

### 4.2. DNA extraction

DNA was extracted from 0.5 g field soil samples using the ultraclean DNA extraction kit (MoBio, USA) according to the manufacturer's instructions. The DNA concentrations were determined in a biophotometer (Eppendorf, Germany) by measuring absorbance at 260 nm (A260), assuming that 1 A260 unit corresponds to 50 ng of DNA per µl. DNA extraction was further confirmed by electrophoresis on a 1% agarose gel. The extracted DNA was dissolved in 50 µl TE buffer and stored at − 20 °C until further analysis.

### 4.3. Real time PCR quantification of eubacteria, actinomycetes and methanotrophs

Real time PCR was performed on a Step one plus real time PCR (ABI, USA) to quantify the representative microbial species. Reaction mixture prepared with 2 µl of DNA template, 10 µl of 2X SYBR green master mix (Affymetrix, USA), 200 nM of primer (GCC Biotech, N Delhi). Final volume of PCR reaction mixture was made to 20 µl with PCR grade water (MP Bio, USA). Primers targeting 16S rRNA gene of eubacteria, 16S rRNA gene of actinomycetes and pmoA gene (particulate methane monooxygenase) of methanotrophs were used to quantify the microbial abundance. The primers (5′-3′) for eubacteria were 1F (CCT ACG GGA GGC AGC AG) and 518R (ATT ACC GCG GCT GCT GG) (Baek et al., 2010); actinomycetes Act235 F (CGC GGC CTA TCA GCT TGT TG) and Act878R (CCG TAC TCC CCA GGC GGG G) (Jafari et al., 2014). The primers for pmoA were A189F (5- GGN GAC TGG GAC TTCT GG-3) and mb661R (5- CCG GMG CAA CGT CYT TAC C-3). This primer set targets methanotrophs covering both type I and II including *Methylobacter or Methylosarcina, Methylococcus, Methylosinus* group, *Methylocapsa, Nitrosococcus* (Mohanty et al., 2017). Quantification of microbial genes was carried out by real time PCR approach targeting the functional groups (Kolb, 2009). Thermal cycling was carried out by an initial denaturizing step at 94 °C for 4 min, 40 cycles of 94 °C for 1 min, target specific annealing temperature for 30 s, 72 °C for 45 s; final extension carried out at 72 °C for 5 min. Annealing temperature for 16S rRNA of bacteria was 50 °C, 16S rRNA of actinomycetes was 52 °C and for actinomycetes was 55 °C. Fluorescence was measured during elongation step. Data analysis was carried out with Step one plus software (ABI, USA) as described in user's manual. The cycle at which the fluorescence of target molecule number exceeded the background fluorescence (threshold cycle [$C_T$]) was determined from dilution series of target DNA with defined target molecule amounts. $C_T$ was proportional to the logarithm of the target molecule number. The quality of PCR amplification products were determined by melting curve analysis with temperature increase of 0.3 °C per cycle. Standard for the genes was made from series of 10 fold dilutions of purified amplified products and data presented as number of cells per gram of soil.

#### 4.3.1. Statistical analyses

All statistical analyses were carried out using the "agricolae" and "vegan" packages of the statistical software R (2.15.1) (Ihaka and Gentleman, 1996). Results for the experiments were presented as arithmetic means and standard deviation of triplicate observations. Tukeys honestly significant difference (HSD) test was performed to define the significant difference among treatments at α 0.05. Effect of factors (temperature, moisture holding capacity, biochar and chlorpyrifos) on the variables ($CH_4$ oxidation, $CO_2$ production, abundance of eubacteria, actinomycetes and methanotrophs), was tested by analysis of variance (ANOVA) at α 0.05. Estimated data were ln transformed for ANOVA. Linear regression models were developed to predict $CH_4$ oxidation from different variables.

## 5. Results

### 5.1. Gross $CH_4$ consumption

Methane consumption under the influence of temperature, moisture, biochar and chlorpyrifos is depicted in Fig. 1. $CH_4$ consumption progressed with initial lag phase followed by rapidly declining log phase, indicating classical methanotrophic activity (Horz et al., 2002). $CH_4$ consumption was slow at 15 °C compared to 35 °C and 45 °C. Chlorpyrifos inhibited $CH_4$ consumption, while biochar stimulated $CH_4$ consumption. Increasing soil moisture from 60% to 100% also stimulated $CH_4$ consumption. Apparent $CH_4$ consumption rate k of different treatment was estimated to exhibit the extent of $CH_4$ consumption under the influence of different factors (Table 1). $CH_4$ consumption rate k varied from 0.065 ± 0.012 to 0.608 ± 0.014. Lowest k value was in the soil incubated at 15 °C with no biochar- 60% MHC-10 ppm chlorpyrifos. Highest rate k was in the treatment of 35C-1% biochar-100% MHC-0 ppm chlorpyrifos. The negative effect of chlorpyrifos on $CH_4$ consumption peaked at 35 °C. For example, at 35 °C chlorpyrifos reduced the k value by 67% at 60% MHC and 50.88% at 100% MHC. Soil moisture decreased the toxicity of chlorpyrifos. Biochar stimulated $CH_4$ consumption by 44% at 15 °C, 61% at 35 °C, and 51% at 45 °C. Biochar amendment alleviated the negative effect chlorpyrifos on $CH_4$ consumption. The antagonistic effect of chlorpyrifos was alleviated by 23–25% at 15 °C and by 27% at 35 °C and 45 °C.

### 5.2. Gross $CO_2$ production

Production of $CO_2$ from soil was estimated as the cumulative $CO_2$ in the headspace of the vials. In general $CO_2$ production followed the trend of $CH_4$ consumption (Table 1). Highest amount of $CO_2$ was produced at 35 °C and lowest at 15 °C. Biochar and soil moisture stimulated $CO_2$ production. Chlorpyrifos inhibited $CO_2$ production at 60% MHC. However, with biochar and 100% MHC $CO_2$ production was not significantly inhibited in most of the treatments. Headspace $CO_2$ concentration ($ng\,g^{-1}$ soil) ranged from 446.09 ± 15.06 to 1989.97 ± 116.71. Lowest was in the treatment of 15 °C-no biochar-60% MHC-10 ppm chlorpyrifos. Highest $CO_2$ production was in the treatment of 35 °C-1% biochar-100% MHC-0 ppm chlorpyrifos.

### 5.3. Microbial abundance

Temperature, moisture, biochar stimulated the abundance of pmoA gene of methanotrophs and 16S rRNA of eubacteria (Table 2). Abundance of pmoA gene ($\times 10^5$ $g^{-1}$ soil) varied from 1.23 ± 0.58 to 30.67 ± 7.51. Abundance of total eubacteria ($\times 10^6$ $g^{-1}$ soil) varied from 2.33 ± 0.58 to 85.67 ± 7.00. Lowest abundance of these two

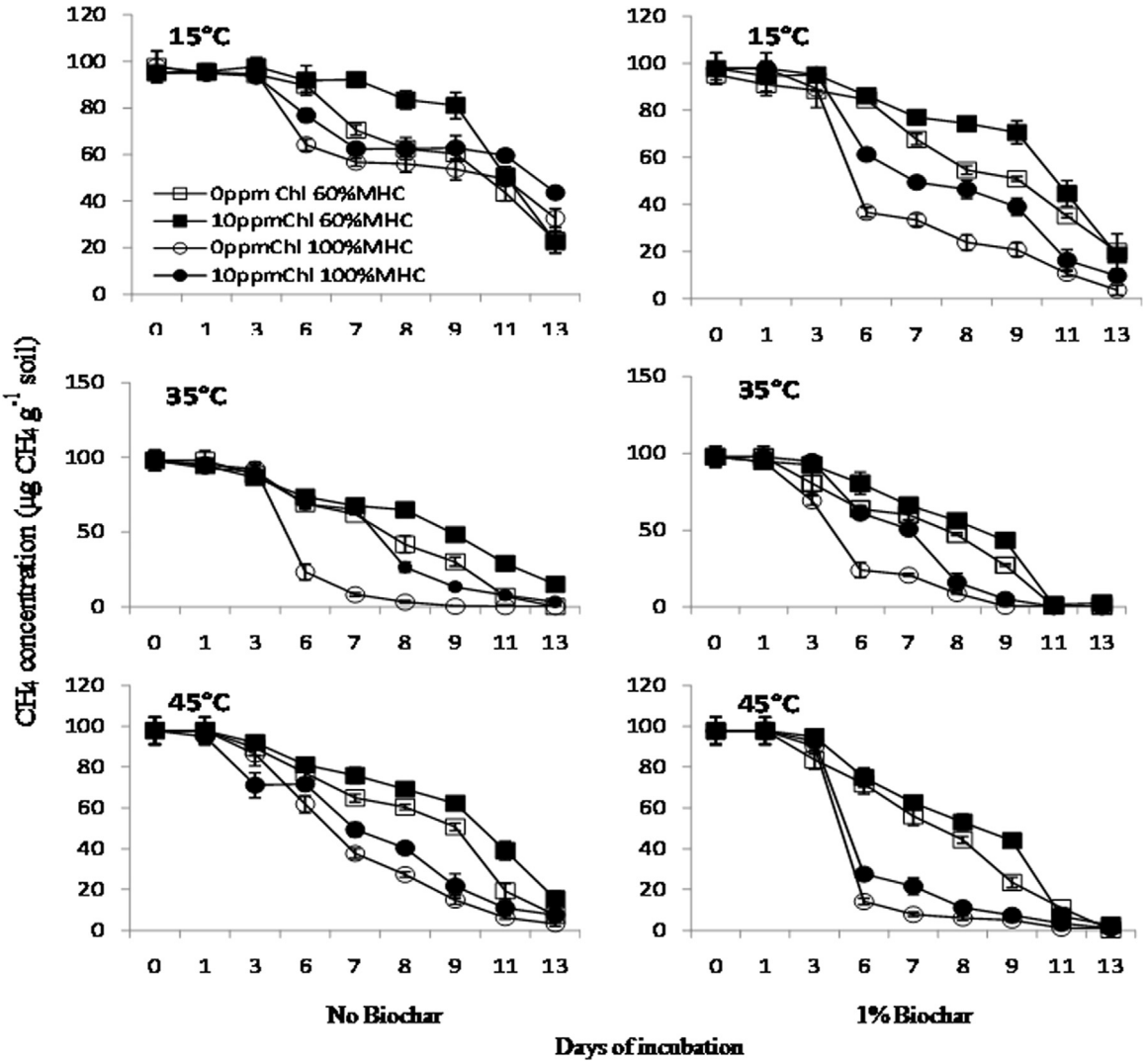


**Fig. 1.** Consumption of $CH_4$ in soil under the interactive influence of different factors. The factors were chlorpyrifos (Chl) concentration (0 ppm or 10 ppm), temperature (15 °C, 35 °C or 45 °C). biochar concentration (no biochar or 1% biochar), and moisture holding capacity (MHC) (60% or 100%). Plots on left panel represent treatments of no biochar and on right of 1% biochar. Y axis represents $CH_4$ concentration in the headspace of microcosms and X axis represent incubation period (in days). Each data point represents arithmetic mean and error bars are standard deviation of three replicated observations.

groups was in the treatment of 15 °C-no biochar-60% MHC and 10 ppm chlorpyrifos. Highest value of these bacterial groups was in the treatment of 35 °C-1% biochar-60% MHC and no chlorpyrifos. Temperature influenced pmoA and eubacteria as 35 °C > 45 °C > 15 °C. Biochar (1%) and soil moisture (100% MHC) stimulated abundance of these two microbial groups irrespective of treatment. Chlorpyrifos inhibited the abundance of pmoA and 16S rRNA genes. Actinomycetes population ($\times 10^4$ $g^{-1}$ soil) varied from 7.67 ± 1.53 to 57.33 ± 6.66. Abundance of actinomycetes followed as 35 °C > 45 °C > 15 °C. Both biochar and soil moisture stimulated actinomycetes. Unlike the eubacteria and methanotrophs, actinomycetes abundance was stimulated by chlorpyrifos. Lowest abundance of actinomycetes was in the treatment of 15 °C-60% MHC-0% biochar and 0 ppm chlorpyrifos. Highest abundance of actinomycetes was in the treatment of 35 °C-1% biochar-100% MHC-10 ppm chlorpyrifos.

### *5.4. Interactive effect of climate variables, chlorpyrifos and biochar on $CH_4$ consumption*

The effect of temperature, biochar, soil moisture, and chlorpyrifos on different parameters was evaluated by ANOVA (Table 3). Parameters were $CH_4$ consumption rate, $CO_2$ production and microbial abundance. All the factors at individual level influenced significantly to the parameters ($p < 0.01$). Low p value indicated significant impact of the factors on the variables. In most of the cases the interaction among the factors had no significant effect on the parameters. The relation between $CH_4$ oxidation and different parameters evaluated by regression analysis. Linear regression models indicated significant positive relation between $CH_4$ oxidation with $CO_2$ production ($R^2$ 0.799), pMOA gene copies of methanotrophs ($R^2$ 0.810), 16S rRNA gene copies of eubacteria ($R^2$ 0.772). However, no significant relation observed with the 16S rRNA gene copies actinomycetes ($R^2$ 0.147). Regression models also predicted $CH_4$ consumption rate as $0.0001 \times CO_2$-0.1 ($p < 0.0001$) (Fig. 2). Similarly, $CH_4$ consumption rate was predicted as 0.015 × pmoA genes + 0.067 ($p < 0.0001$), and 0.006 × 16S rRNA genes of bacteria + 0.081 ($p < 0.0001$).

## 6. Discussion

The temporal variation of $CH_4$ consumption proceeded with a initial lag phase followed by the rapidly declining log phase, due to the microbial growth and activity. This trend of methanotrophic activity in the soil agrees with our previous studies (Kollah et al., 2014). The relation between temperature and $CH_4$ consumption was not linear

**Table 1**
Effect of chlorpyrifos on the rate of $CH_4$ oxidation of soil. Each value represents arithmetic mean ± standard deviation of three replicated observations. Values followed by the same letter within a column are not significantly different.

| Temperature (°C) | Biochar (%) | Soil moisture holding capacity (%) | Chlorpyrifos (ppm or μg $g^{-1}$ soil) | Apparent $CH_4$ consumption rate k (ng $CH_4$ consumed $g^{-1}$ soil $d^{-1}$) | Cumulative $CO_2$ production (ng $g^{-1}$ soil) |
|---|---|---|---|---|---|
| 15 | 0 | 60 | 0 | 0.110 ± 0.037$^{k}$ | 575 ± 22.61$^{j}$ |
| | | | 10 | 0.065 ± 0.012$^{m}$ | 446 ± 15.06$^{k}$ |
| | | 100 | 0 | 0.084 ± 0.038$^{l}$ | 636 ± 15.20$^{i}$ |
| | | | 10 | 0.085 ± 0.005$^{l}$ | 604 ± 11.27$^{i}$ |
| | 1 | 60 | 0 | 0.152 ± 0.029$^{j}$ | 955 ± 75.15$^{g}$ |
| | | | 10 | 0.116 ± 0.014$^{k}$ | 675 ± 56.15$^{i}$ |
| | | 100 | 0 | 0.300 ± 0.038$^{g}$ | 1164 ± 153.67$^{f}$ |
| | | | 10 | 0.226 ± 0.006$^{h}$ | 1024 ± 13.76$^{g}$ |
| 35 | 0 | 60 | 0 | 0.435 ± 0.002$^{d}$ | 1136 ± 3.62$^{f}$ |
| | | | 10 | 0.143 ± 0.008$^{j}$ | 1070 ± 10.39$^{g}$ |
| | | 100 | 0 | 0.590 ± 0.018$^{a}$ | 1412 ± 9.81$^{d}$ |
| | | | 10 | 0.299 ± 0.019$^{g}$ | 1147 ± 10.15$^{f}$ |
| | 1 | 60 | 0 | 0.507 ± 0.062$^{c}$ | 1714 ± 38.88$^{b}$ |
| | | | 10 | 0.370 ± 0.072$^{f}$ | 1360 ± 262.60$^{e}$ |
| | | 100 | 0 | 0.608 ± 0.014$^{a}$ | 1989 ± 116.71$^{a}$ |
| | | | 10 | 0.576 ± 0.042$^{b}$ | 1750 ± 67.29$^{b}$ |
| 45 | 0 | 60 | 0 | 0.198 ± 0.021$^{i}$ | 915 ± 61.01$^{h}$ |
| | | | 10 | 0.133 ± 0.015$^{j}$ | 876 ± 22.75$^{h}$ |
| | | 100 | 0 | 0.301 ± 0.011$^{g}$ | 1106 ± 24.05$^{f}$ |
| | | | 10 | 0.223 ± 0.029$^{h}$ | 912 ± 36.17$^{g}$ |
| | 1 | 60 | 0 | 0.401 ± 0.017$^{e}$ | 1505 ± 86.40$^{d}$ |
| | | | 10 | 0.293 ± 0.008$^{g}$ | 1388 ± 135.35$^{e}$ |
| | | 100 | 0 | 0.428 ± 0.064$^{e}$ | 1578 ± 206.36$^{c}$ |
| | | | 10 | 0.383 ± 0.025$^{f}$ | 1593 ± 154.08$^{c}$ |
| Tukeys HSD (α 0.05, df error 71) | | | | 0.32 | 87.82 |

Soils were amended with chlorpyrifos (0 ppm or 10 ppm) maintained under two moisture holding capacity (60% or 100%) and incubated at temperature of 15 °C, 35 °C, or 45 °C.

**Table 2**
Effect of chlorpyrifos on the abundance of genes representing Eubacteria, Methanotrophs and Actinomycetes. Each value represents arithmetic mean ± standard deviation of three replicated observations. Values followed by the same letter within a column are not significantly different.

| Temperature (°C) | Biochar (%) | Soil moisture holding capacity (%) | Chlorpyrifos (ppm or μg $g^{-1}$ soil) | Methanotrophs pmoA genes (× $10^5$ $g^{-1}$ soil) | Heterotrophic Bacterial 16S rRNA genes (× $10^6$ $g^{-1}$ soil) | Actinomycetes 16S rRNA genes (× $10^4$ $g^{-1}$ soil) |
|---|---|---|---|---|---|---|
| 15 | 0 | 60 | 0 | 2.00 ± 1.00$^{k}$ | 4.67 ± 0.58$^{j}$ | 7.67 ± 1.53$^{k}$ |
| | | | 10 | 1.23 ± 0.58$^{k}$ | 2.33 ± 0.58$^{k}$ | 11.33 ± 0.58$^{j}$ |
| | | 100 | 0 | 10.33 ± 2.08$^{h}$ | 8.57 ± 0.58$^{i}$ | 12.67 ± 2.08$^{j}$ |
| | | | 10 | 5.00 ± 1.00$^{j}$ | 6.00 ± 1.00$^{j}$ | 16.67 ± 1.15$^{h}$ |
| | 1 | 60 | 0 | 4.00 ± 1.00$^{j}$ | 9.33 ± 0.58$^{h}$ | 31.33 ± 1.53$^{e}$ |
| | | | 10 | 1.33 ± 0.59$^{k}$ | 7.33 ± 0.58i | 46.33 ± 1.53$^{c}$ |
| | | 100 | 0 | 12.33 ± 1.53$^{g}$ | 12.33 ± 0.58$^{h}$ | 35.33 ± 1.15$^{d}$ |
| | | | 10 | 6.00 ± 2.00$^{i}$ | 5.00 ± 1.00$^{j}$ | 53.33 ± 6.66$^{b}$ |
| 35 | 0 | 60 | 0 | 22.67 ± 2.08$^{c}$ | 55.00 ± 6.00$^{c}$ | 17.00 ± 1.00$^{h}$ |
| | | | 10 | 13.67 ± 1.53$^{f}$ | 32.67 ± 2.08$^{g}$ | 26.67 ± 4.16$^{f}$ |
| | | 100 | 0 | 31.67 ± 3.51$^{a}$ | 67.00 ± 2.00$^{b}$ | 22.67 ± 2.08$^{g}$ |
| | | | 10 | 20.33 ± 2.08$^{d}$ | 36.67 ± 4.51$^{f}$ | 39.00 ± 3.61$^{cd}$ |
| | 1 | 60 | 0 | 30.67 ± 7.51$^{b}$ | 85.67 ± 7.00$^{a}$ | 32.33 ± 1.53$^{e}$ |
| | | | 10 | 19.67 ± 10.79$^{d}$ | 45.33 ± 8.26$^{d}$ | 38.00 ± 1.00$^{cd}$ |
| | | 100 | 0 | 34.33 ± 0.51$^{a}$ | 84.67 ± 5.77$^{a}$ | 43.00 ± 1.00$^{c}$ |
| | | | 10 | 28.67 ± 0.58$^{b}$ | 56.33 ± 1.53$^{c}$ | 57.00 ± 4.58$^{a}$ |
| 45 | 0 | 60 | 0 | 14.00 ± 1.73$^{e}$ | 35.33 ± 3.51$^{f}$ | 11.00 ± 1.00$^{f}$ |
| | | | 10 | 10.00 ± 1.00$^{h}$ | 29.00 ± 1.00$^{g}$ | 15.67 ± 2.08$^{i}$ |
| | | 100 | 0 | 10.33 ± 0.58$^{h}$ | 43.67 ± 3.79$^{e}$ | 13.67 ± 1.53$^{i}$ |
| | | | 10 | 8.33 ± 0.58$^{i}$ | 35.00 ± 2.65$^{f}$ | 17.67 ± 0.58$^{h}$ |
| | 1 | 60 | 0 | 18.33 ± 0.58$^{d}$ | 49.33 ± 5.13$^{d}$ | 19.33 ± 4.16$^{g}$ |
| | | | 10 | 14.33 ± 3.21$^{e}$ | 29.33 ± 4.62$^{g}$ | 34.00 ± 5.57$^{d}$ |
| | | 100 | 0 | 18.33 ± 0.58$^{d}$ | 55.67 ± 3.06$^{c}$ | 27.33 ± 2.08$^{f}$ |
| | | | 10 | 16.33 ± 1.53$^{e}$ | 45.33 ± 2.52$^{d}$ | 33.67 ± 1.53$^{e}$ |
| Tukeys HSD (at α 0.05, df error 71) | | | | 2.05 | 3.01 | 2.84 |

Soils were amended with chlorpyrifos (0 ppm or 10 ppm) maintained under two moisture holding capacity (60% or 100%) and incubated at temperature of 15 °C, 35 °C, or 45 °C.

**Table 3**
Analysis of variance (ANOVA) to elucidate the effect of different factors on the parameters of the study. The p values of individual or multiple factors demonstrate the interactive effect. A sign(s) followed by p values represents the level of significance.

| Factors | $CH_4$ consumption | $CO_2$ production | Heterotrophs 16S rRNA gene | Methanotrophs pmoA gene | Actinomycetes 16S rRNA gene |
|---|---|---|---|---|---|
| Temperature (T) | < 0.0001*** | < 0.0001*** | < 0.0001*** | < 0.0001*** | < 0.01* |
| Biochar (B) | < 0.0001*** | < 0.0001*** | < 0.001** | < 0.01* | < 0.0001*** |
| Soil moisture (M) | < 0.001** | < 0.0001*** | < 0.01* | < 0.01* | < 0.0001*** |
| Chlorpyrifos (C) | < 0.0001*** | < 0.001** | < 0.0001*** | < 0.001** | < 0.0001*** |
| T × B | > 0.1 | > 0.1 | > 0.1 | > 0.1 | < 0.0001*** |
| T × M | > 0.1 | > 0.1 | > 0.1 | > 0.1 | > 0.1 |
| B × M | < 0.01* | > 0.1 | > 0.1 | > 0.1 | > 0.1 |
| T × C | < 0.01* | < 0.01* | > 0.1 | < 0.01* | > 0.1 |
| B × C | > 0.1 | > 0.1 | > 0.1 | > 0.1 | > 0.1 |
| M × C | > 0.1 | > 0.1 | > 0.1 | > 0.1 | > 0.1 |
| T × B × M | > 0.1 | > 0.1 | > 0.1 | > 0.1 | > 0.1 |
| T × B × C | > 0.1 | > 0.1 | > 0.1 | > 0.1 | > 0.1 |
| T × M × C | > 0.1 | > 0.1 | > 0.1 | > 0.1 | > 0.1 |
| B × M × C | > 0.1 | > 0.1 | > 0.1 | > 0.1 | > 0.1 |
| T × B × M × C | > 0.1 | > 0.1 | > 0.1 | > 0.1 | > 0.1 |

Signif. codes: 0.0001 '***' 0.001 '**' 0.01 '*' 0.05 '.'.
The factors were temperature (15 °C, 35 °C, 45 °C), soil moisture holding capacity (60%, 100%), biochar (0%, 1%) and chlorpyrifos (0 ppm, 10 ppm). The parameters were $CH_4$ consumption rate k, cumulative $CO_2$ production, abundance of 16S rRNA gene of eubacteria, actinomycetes, and pmoA gene of methanotrophs.

because the CH4 consumption rate was highest at the 35 °C followed by 45 °C and 15 °C. $CH_4$ consumption found to be optimal at 25–35 °C in many ecosystems like neutral landfill cover soil (Boeckx and Vancleemput, 1996), soil (Crill and Silvola, 1994), and arctic Alaskan lakes (Lofton et al., 2014). In a study with temperate forest soil and rice field soil, maximum $CH_4$ consumption was at 25–35 °C, where the $CH_4$ consumption was severely inhibited at < 15 °C (Peltoniemi et al., 2016). The present study suggested that 35 °C was the optimum temperature for high $CH_4$ consumption in the Vertisol. Thus, the climate warming is expected to stimulate methanotrophic activity in Vertisol until the atmospheric temperature is near 35 °C.

The soil used in the experiment is from the tropical zone where temperature in summer sometime goes as high as 45 °C. At high temperature (45 °C) $CH_4$ consumption rate varied from 0.133 to 0.428 ng $g^{-1}$ soil $d^{-1}$ indicating activity of methanotrophs at high temperature. In a study on the $CH_4$ consumption in soils of Northern Europe was estimated and found that $CH_4$ consumption rate varied from about 0.5–165 ng $CH_4$ $g^{-1}$ $d^{-1}$ (Dobbie et al., 1996). Thus, the $CH_4$ consumption rate estimated in this study was in low range. There was high $CH_4$ consumption under flooded condition which could be due to the homogenous mixing of nutrients in slurries. Shaking also enhanced the mixing of soil with the headspace $O_2$ and $CH_4$. Probably the

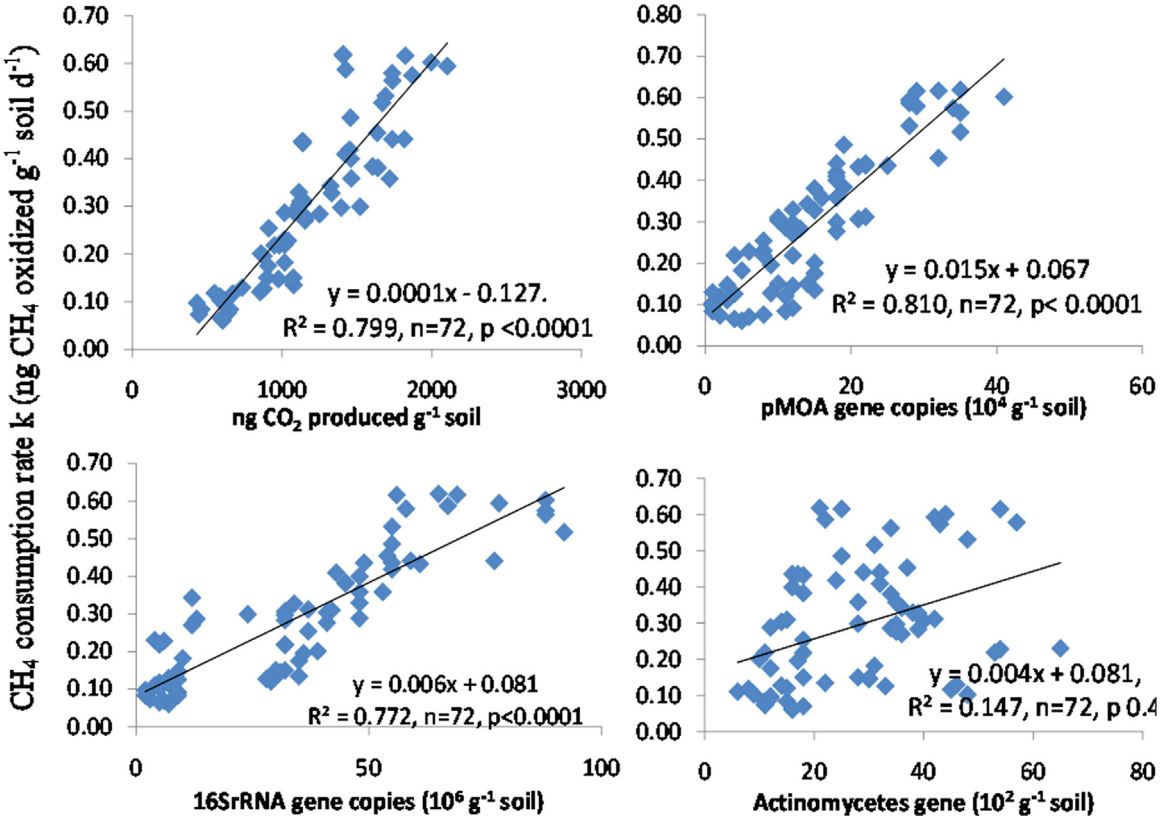


**Fig. 2.** Linear regression models of $CH_4$ consumption and abundance of 16S rRNA gene of eubacteria, pmoA gene of methanotrophs, 16S rRNA of actinomycetes and $CO_2$ production. Y axis represents $CH_4$ consumption rate k and X axis represents different parameters.

homogenous condition favoured methanotrophs and stimulated $CH_4$ consumption. In addition, to aerobic $CH_4$ consumption, partial anaerobic $CH_4$ oxidation in flooded soil might have stimulated the $CH_4$ consumption. Contrastingly, soil prepared at 60% MHC had clods. The permeability of gas phase ($O_2$ and $CH_4$) into these clods could be low limiting $CH_4$ oxidation.

Biochar (BC) stimulated $CH_4$ consumption irrespective of treatments. Similar results has been reported earlier (Yu et al., 2013). Significant positive correlation ($p < 0.0001$) between methanotrophic bacteria and k values indicated that, BC stimulated $CH_4$ consumption by proliferating the methane oxidizing bacteria. The mechanism of such unusual degree of enduring soil response can be explained by multiple positive effects on soil properties. For example, biochar improves soil properties like pH (in case of acidic soils, since it is typically alkaline), increases water retention, increases nutrients availability, enhances cation exchange capacity (CEC) of the soil, enhances microbial communities, and benefits soil physical structure through increasing porosity (Atkinson et al., 2010). Abundance of bacteria and mycorrhizae fungi in BC surface have been reported, defining the positive linkage between surface area and microbial activities (Steinbeiss et al., 2009). Biochar sorbs inorganic nutrients as well as organic substances and gases (like $CH_4$ and $O_2$) (Subedi et al., 2013) and probably extended favourable environment for methane-consuming bacteria.

Chlorpyrifos was applied at 10 ppm level, equivalent to a dose of 5 kg active ingredients (a.i) per ha. This concentration was within the range of field doses recommended for different crops (Posey et al., 2006). Abundance of eubacteria, methanotrophs and actinomycetes was estimated in this study. Their abundance was in medium range. Chlorpyrifos inhibited the abundance of eubacteria and methanotrophic bacteria but stimulated actinomycetes. Chlorpyrifos and its metabolites has been reported to inhibit wide range of bacteria including free living aerobic nitrogen fixing bacteria, symbiotic nitrogen fixing bacteria, and nitrifiers as described earlier. Chlorpyrifos inhibits bacterial enzymes including hydrolases (carboxylesterase, acid phosphatase, β-glucosidase, urease and protease) and oxidoreductases (dehydrogenase and catalase) (Sanchez-Hernandez et al., 2017).

Eubacterial abundance represent the overall bacterial activity of soil. Many heterotrophic bacteria are known to biodegrade organic pollutants including chlorpyrifos. For example, *Acinetobacter*, *Enterobacter* degrade chlorpyrifos in soil (Singh et al., 2004). Methanotrophs were enumerated as they are mainly responsible for soil's $CH_4$ uptake. Actinomycetes were enumerated as they degrade the insecticide and proliferate under stress condition (Etesami and Beattie, 2017). Abundance of both eubacteria and methanotrophs varied concurrently with the $CH_4$ consumption and $CO_2$ production. Methanotrophs are also part of the large eubacterial community of soil. However, the abundance of actinomycetes varied oppositely with the heterotrophs and methanotrophs. Probably, the stress conditions arising from chlorpyrifos, moisture, and temperature inhibited heterotrophs and methanotrophs stimulated actinomycetes. Therefore, chlorpyrifos inhibited methanotrophic bacteria and eubacteria but stimulated actinomycetes.

Soil moisture alleviated the inhibitory effect of the chlorpyrifos. Adsorption of chlorpyrifos on biochar could be high at 100% MHC. Therefore, the chlorpyrifos was readily available for biodegradation. Biodegradation of chlorpyrifos could be higher at 100% MHC than at 60% MHC. This was supported with the microbial abundance data. High microbial abundance was found at 100% MHC than the 60% MHC. Many heterotrophic bacteria degrade chlorpyrifos. However, this study did not indicate involvement of heterotrophs in chlorpyrifos degradation as their population declined in soil treated with chlorpyrifos. Biochar stimulated the methanotrophic activity by stimulating microbial activities. This could be due to multiple beneficial effect of biochar those favoured $CH_4$ oxidation. Properties like high surface area, porous structure, high CEC offered stimulated methanotrophs (Subedi et al., 2013). High CEC of biochar enables it to adsorb anionic nutrients. For example, high $NH_4$ adsorption to the surface of biochar potentially stimulates the nitrifiers population. Many nitrifiers also oxidize $CH_4$.

Impact of the factors on $CH_4$ consumption was estimated by ANOVA, which suggested that the factors (temperature, biochar, soil moisture, and chlorpyrifos) significantly influenced the parameters ($CH_4$ consumption, $CO_2$ production, and microbial abundance). However, the interaction of the factors in most cases had no significant effect on the parameters. It could be due to unrelated trend in the effect of factors. For example, increasing the temperature inhibited $CH_4$ consumption, while the amendment of biochar stimulated $CH_4$ consumption. Therefore, the interaction between temperature and biochar ($T \times B$) had no significant effect on the $CH_4$ consumption. In other hand biochar × moisture ($B \times M$) had significant positive effect ($p < 0.01$) on the $CH_4$ consumption as both the factors individually stimulated $CH_4$ consumption.

## 7. Conclusive remarks

Experiment outlined the effect of climate change factors (temperature, moisture), chlorpyrifos, and biochar on $CH_4$ consumption. $CH_4$ consumption was highest at 35 °C followed by 45 °C and 15 °C. Moisture at 100% enhanced $CH_4$ consumption than at 60% MHC. Biochar stimulated $CH_4$ consumption irrespective of treatment. The trend of $CH_4$ consumption and $CO_2$ production in the treatments varied similarly. The abundance of eubacteria and methanotrophs varied similarly with $CH_4$ oxidation and $CO_2$ production. However, abundance of Actinomycetes varied in opposite trend with that of the methanotrophs. Probably, the actinomycetes abundance was stimulated by chlorpyrifos. Study concluded that intensive use of chlorpyrifos under future climate change may adversely affect soil $CH_4$ uptake leading to severity of climate change. Although biochar can be used to improve $CH_4$ consumption in soil contaminated with chlorpyrifos. However, the effectiveness of biochar can be adversely affected by temperature extremes.

## Acknowledgement

This research was made possible by financial support from the Department of Biotechnology (DBT) Govt. of India for the project “Biodegradation of pesticides under changing climate and metagenomic profiling of functional microbes (Bio-CARe/06/175/2011-12)”. Authors declare no conflict of interest of any type.

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